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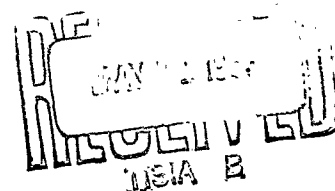
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ISOLATION OF THE POLYHEDER VIRUS AND THE NATURE OF POLYHEDERS

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ISOLATION OF THE POLYHEDER VIRUS AND THE NATURE OF POLYHEDERS

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Zeitschrift fuer Naturforschung,
Vol 2b, 1947, pages 122-143.

In the infectious polyhedr disease of insect larvae, polyhedral protein crystals form in the nuclei of almost all cells of the body of the insects affected. The nature of these polyheders was investigated on P. dispar, L. monacha, and B. mori larvae. They were found to consist to the major part of a non-infectious, very uniform polyhedr protein with a molecular weight of 276,600, 336,000, or 378,000 and to a small extent of an infectious polyhedr virus protein with a particle weight of about 10^6 . These infectious polyhedr virus particles, which are rich in nucleic acids, could be isolated in a pure state. Electron-microscopic pictures of them could then be taken and their sedimentation constants and diffusion constants could be measured. The polyhedr protein and virus protein could be split into smaller components which then reunited to form high-molecular particles. The polyhedr protein and the polyhedr virus were found to be related serologically.

The polyhedr disease affects the larvae of certain species of moths. Investigation of this disease formed the subject of a considerable amount of research carried out already in the past century, because this infectious disease is the main factor by which the widespread occurrence of some of our principal forest and field pests such as L. monacha, P. dispar, D. pini is checked. The disease, which is almost always lethal, has been named after the polyhedr-shaped crystal formations that are 1-10 μ large and occur in the nuclei of almost all cells of the body of the insects. A comprehensive account of the knowledge on the subject acquired before and of the problems involved in this disease, which is of great importance from the economic standpoint, has been given in an earlier article [1], so that we shall only report on research carried out during the past four years.

Experiments carried out on insects showed that the polyheders themselves are etiologically related to the disease and that they do not merely represent non-infectious reaction products derived from the cell nucleus. Starting from the known fact that the polyheders are soluble in weak alkalis and that the middle intestine of the larvae which are susceptible to the polyheder virus infection has an alkaline reaction, solutions of the polyheders in weak alkali were investigated by the ultracentrifuge method and electrophoretically. It was found that after the polyheders had been dissolved under definite conditions and this solution has been purified, centrifuging resulted in the separation of a uniform protein with a constant molecular weight and a constant electric charge. The molecular weight was approximately 200,000-300,000 [1,2,3]. The infectious dose of the polyheder solutions was according to some determinations 10^{-10} g. of protein per individual for silkworms (Bm = *B. mori*), 10^{-13} g. for *Porthetria dispar* (Pd), and 10^{-15} g. for *Lymantria monacha* (Lm). This high virulence, which had been established in an inadequate number of animal experiments, was not readily reproducible and showed strong fluctuations. Because the minimum infectious dose did not change significantly after 3 hours of centrifuging at 30,000 rpm (60,000 g), the conclusion was drawn that the characteristic protein was uniform and that it presumably was identical with the infectious protein. An admixture of some other high-molecular protein could not have been present in a quantity exceeding 5%. The result obtained in an ultra-filtration experiment indicated that large molecules with a particle weight of several million could not have been present. The isolation of an infectious virus protein with a molecular weight of 200,000-300,000 was of such great fundamental importance that a thorough investigation of the problem became necessary. The investigation had three different aims:

I. Purification in as far as possible, further characterization, and determination of the molecular weight of the polyheder protein and of its components formed by splitting.

II. Improvement of the testing procedure by the use of polyheder-free insects which had been bred for a great number of years, use of a much larger number of insects, isolation of individual insects on which experiments were carried out, and statistical evaluation of the results.

III. A thorough investigation of the question as to whether the polyheder protein is identical with the infectious virus protein.

I. Isolation in the Pure State, Characterization, and Molecular Weight of the Polyheder Protein.

1. Isolation in the Pure State and Characterization.

We had already established before [2,3] that alkaline polyheder solutions behave in a uniform manner in the ultracentrifuge and exhibit sedimentation constants of approximately 10 Svedberg. To the almost

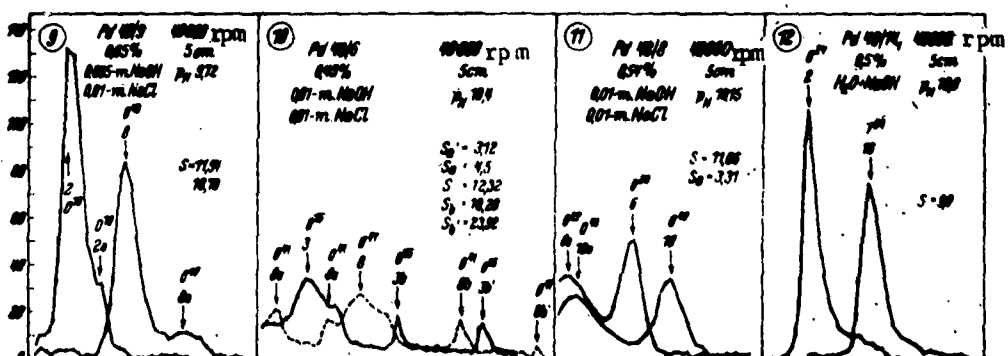
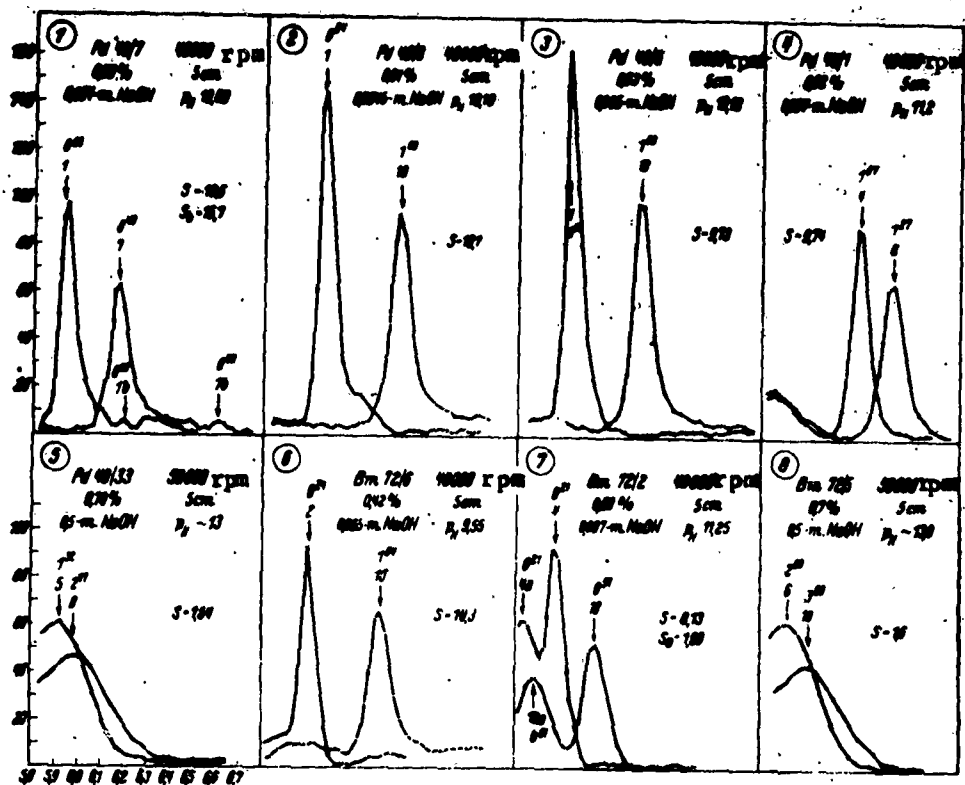
completely white polyheder emulsion an 0.1 N NaOH solution was added in very small drops until the turbid emulsion just about cleared up. The emulsion was first centrifuged twice for 30 min and then once for 15 min at 16,000 rpm (20,000 g) in an Ecco-Blitz centrifuge. As a result of the centrifuging there was sedimentation of a small quantity of brownish impurities. The supernatant light yellow solution was perfectly clear and showed a barely perceptible Tyndall light. If a somewhat smaller quantity of NaOH had been added one could see above the brownish impurities a very thin bluish-white layer of sedimentation. This layer disappeared on addition of more NaOH. The sedimentation constants (s_{20}) of transparent polyheder solutions purified in this manner were then systematically investigated by means of an ultracentrifuge [4] which had been rebuilt according to the scale method. It was established after numerous and difficult experiments that complete solution of the polyheders (to the extent of approximately 90-100% of the quantity weighed in) to form a uniform solution with respect to /molecular/ weight could only be accomplished under definite conditions. These conditions were as follows. For a definite quantity weighed in, e.g., 5 or 7 mg/cm³, a definite initial pH was required [See Note7].

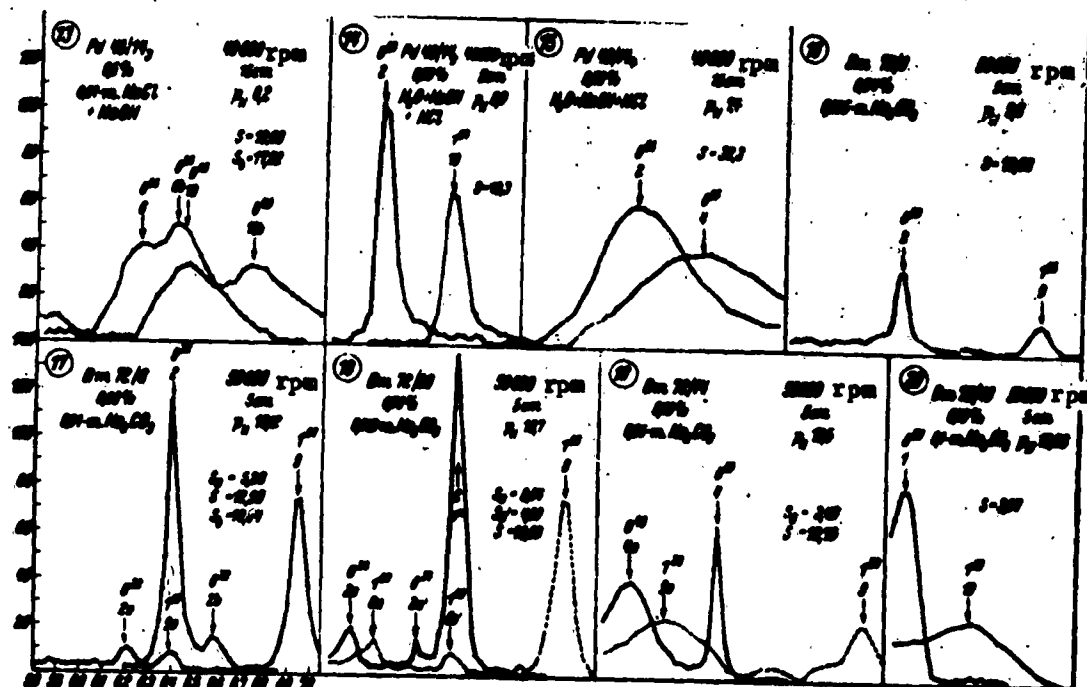
([Note7]: All pH measurements were carried out by means of the following equipment: a low-resistance needle glass electrode from Schott, Iena; a Pehavi-C measurement bridge from Hartmann and Braun, Frankfurt a.M.; and a Multiflex galvanometer MG 3 (2×10^{-9} amp/mm) from B. Lange, Berlin, used as a null point instrument. No amplification was used; the precision was ± 0.03 pH.)

If the hydrogen ion concentration is too low, only a part of the polyheders goes into solution. Furthermore, unstable components with a sedimentation constant of 17-19 Svedberg occur and there are also still heavier components. If the hydrogen ion concentration surpasses a certain value, one encounters in addition to the principal component with approximately 12 Svedberg a less stable component formed by splitting which has a value of 5-6 Svedberg and a more stable component formed by splitting which has a value of 3 Svedberg. Sometimes one gets directly a component with 1.5 Svedberg, which one also obtains in a more or less uniform state on short boiling in an 0.5 N NaOH solution.

Figures 1-5 [See Note7] show this for Pd polyheders (7 mg/cm³) and figures 6-8 for BM polyheders (5 mg/cm³), which behave in an essentially similar manner (this also applies to the Lm polyheders)

([Note7]: In all sedimentation diagrams the changes in position Z are plotted in 1/100 along the axis or ordinates and the values of the radius r are plotted in cm along the axis of abscissae.)





The precise determination of small s_{20} was interfered with greatly by the so-called primary charging effect (Pedersen). Because of the very high salt sensitivity of the polyheder solutions, this effect could not be eliminated readily by addition of a neutral salt. For this reason, systematic tests were carried out to establish the NaCl concentration which does not yet affect the monodispersity. It was established that although a definite quantity of neutral salt that has been added (e.g., 0.01-0.05 M NaCl) increases considerably the rate of dissolution of the polyheders, this addition may easily result in the formation of a component with 17-19 Svedberg (having approximately the double molecular weight of the 12 Svedberg component) and also of components with a higher molecular weight (Figure 9). Furthermore, experimental work with unbuffered salt solutions of a concentration of approximately 0.01 M presents considerable difficulties. Under the circumstances we tried addition in the initial stage of the experiments of 0.1 N NaOH in portions as small as possible to the aqueous polyheder emulsion, carrying out this addition under constant pH control, in order to avoid a pH that was too high (above 11). However, under these conditions the carbon dioxide of the air exerts a buffering effect which is

so high that on addition of 0.01 M NaOH (to give an example) much less protein goes into solution and several components (with a double and four times larger molecular weight) appear (Figure 10). If one compares with this Figure 11, in which under otherwise completely identical conditions the polyheders were covered immediately with a single portion of 0.01 M NaOH, one can see that in this instance the active hydrogen ion concentration was already so high that in addition to the 12-Svedberg component the 3-Svedberg cleavage compound formed in considerable quantities and that furthermore a greater amount of protein went into solution. The exceptional salt sensitivity of the polyheder protein (i.e., the 12-Svedberg compound) can be seen most readily if the Figures 12-15 are compared. Figure 12 shows the s-diagram of an almost completely uniform polyheder protein which has been dissolved in 0.005 M NaOH and dialyzed against a water-NaOH mixture (pH 10.0) with exclusion of the access of air. To this protein solution was added first a quantity of NaCl corresponding to an 0.01 M solution, whereupon the pH dropped to approximately 8.2 (Figure 13). In another instance no NaCl was added, but the solution was brought to a pH 8.0 by adding traces of HCl (Figure 14). In still another experiment, the pH was lowered to 7.4 by means of HCl (Figure 15). One can furthermore see from these experiments that without addition of salt the monodispersity of the polyheder protein is still preserved completely at pH 8 and that only at pH 7.4 aggregation resulting in accumulation of the 32-Svedberg component takes place (this component has a molecular weight which is approximately six times higher than that of the 12-Svedberg component).

A certain reduction of the difficulties involved in solution was achieved after many futile experiments with different bases and buffer solutions such as sodium carbonate-sodium bicarbonate, ammonia-ammonium chloride, different amines (e.g., colamine) in different concentrations it was found that NaOH can be replaced with Na_2CO_3 taken in a definite concentration (Figure 16-20). By this means, a lower initial pH could be applied and the primary charging effect could be suppressed to a considerable extent. This is shown by the higher s_{20} values obtained in comparison with those resulting when NaOH was used (Figures 6-8). Figure 21 shows a somewhat unusual relation between the quantity of polyheder protein that dissolved and the Na_2CO_3 concentration at a constant weighed-in quantity of 7 mg/cm³. It is noteworthy that the optimum Na_2CO_3 concentration for the solution of the polyheders is the same as that required for a uniform occurrence of the 12-Svedberg component. Subsequent experiments showed that when solution is carried out in an 0.004 M Na_2CO_3 solution, addition of 0.05 M NaCl accelerates the dissolution of the polyheders and a uniform dispersion of the protein in the form of the 12-Svedberg component is achieved (Figure 22). To establish this uniformity, the experimental s curve in Figure 22 was supplemented by the theoretical diffusion curve (crosses) determined on the basis of experimentally determined diffusion constants.

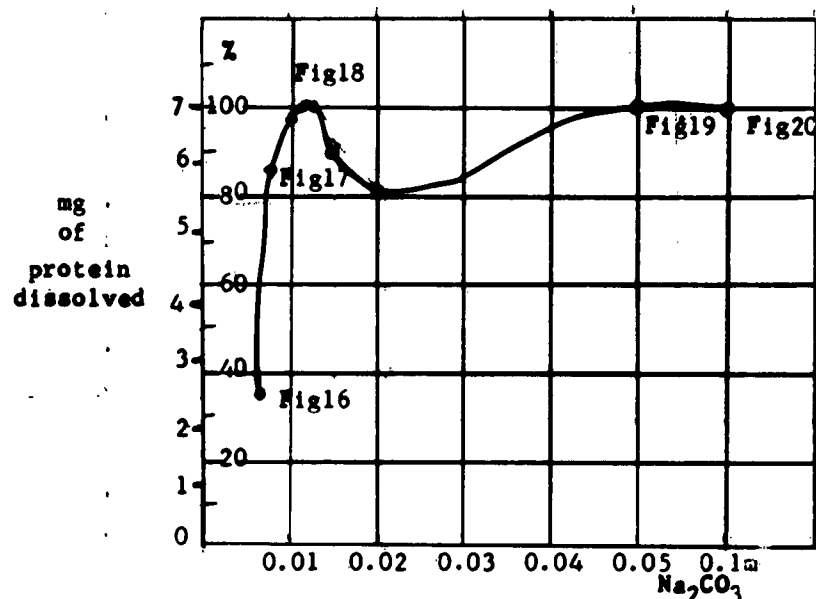


Figure 21. Relation between the quantity of Bm protein dissolved and the concentration of Na₂CO₃.

The almost complete correspondence between the two curves indicates that the polyheder protein was uniform. The dissolution of the polyheders could also be carried out in a mixture of 0.05 M NaHCO₃ and 0.05 M NaCl. The protein could furthermore be dissolved within 12 hours in an 0.1 M NaHCO₃ solution, provided that the polyheders had been ground up thoroughly.

After these preliminary experiments, one could carry out a purification of the polyheder protein from low molecular components which were conceivably present and carry out a precise determination of the sedimentation and diffusion constants of the 12-, 3-, and 1.5-Svedberg components the presence of which had been established. Furthermore, the virus activity of the components could be investigated more closely. As we had already established before [2,3], one can precipitate the polyheder protein isoelectrically by acidifying the solution and then dissolve it again by adding alkali. Repeated recent experiments showed that the polyheder protein which has been precipitated quantitatively by acid or dialysis against distilled water can be dissolved under essentially the same conditions as intact polyheders. It is true that the precipitated protein is more sensitive to an excess of H-ions, i.e., it splits up much more readily, i.e., at lower H-ion concentrations, into the 6-, 3-, or 1.5-Svedberg components. The reason for this is that the intact polyheders contain strongly buffering low-molecular substances. These substances are eliminated during precipitation or dialysis. By redissolving the polyheder proteins, one can also observe how the higher aggregation components

are reconverted into the 12-Svedberg component. Thus, Figure 23 shows the behavior of the protein prepared as shown in Figure 15 after this protein has been precipitated with HCl at pH 6, washed twice with twice distilled water, and redissolved in the form of centrifuged sediment in 0.003 M NaOH. In addition to the 12-Svedberg component, the 17-Svedberg component is now present in a low concentration.

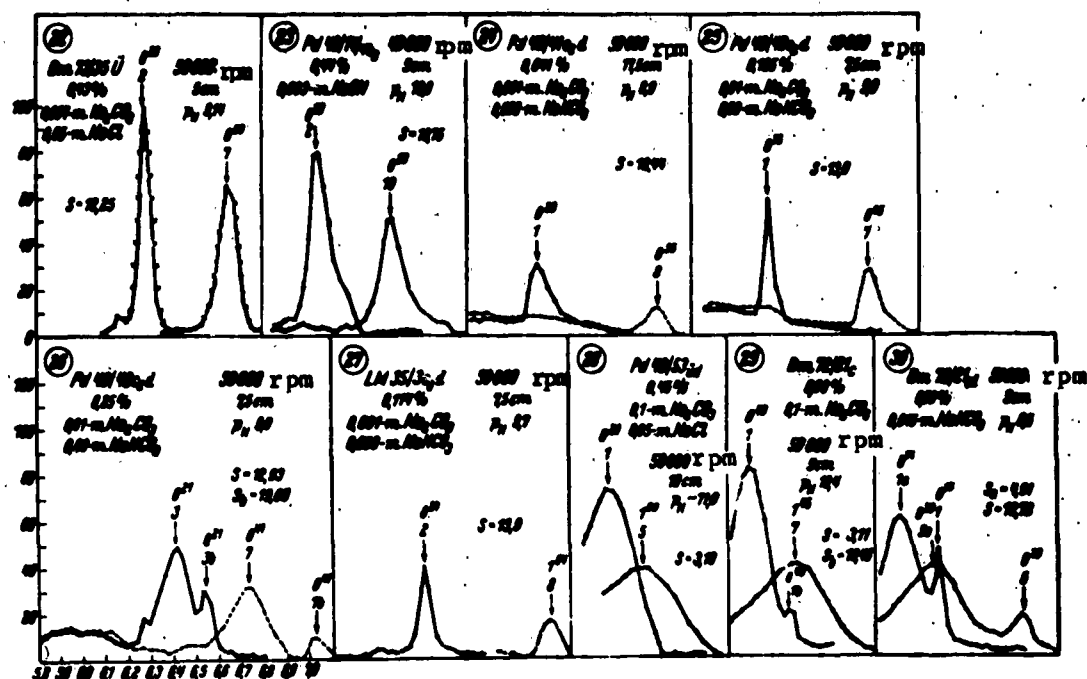
After many experiments, the most advantageous conditions were established under which the precipitated and washed polyheder proteins derived from Bm, Pd, and Lm larvae can be redissolved in order to obtain uniform dispersions containing only the 12-, 3-, or 1.5-Svedberg components (cf. Chapter 2).

2. Determination of the Molecular Weight of the Polyheder Protein and of the Components Formed from it by Splitting.

a) The 12-Svedberg Principal Component

In order to avoid extrapolations which would be uncertain if there is a concentration dependence of the sedimentation constant (s_{20}) and of the diffusion constant (D_{20}), both constants were determined at concentrations which were as low as possible.

The Pd (7 mg/cm³) polyheders were dissolved in 0.00475 M NaOH for experiments Pd 40c₂d and 41c₂d and in 0.005 M NaOH for the experiments 43c₂d, 44c₂d and 44c₂dd. Centrifuging for 15-30 min. at 16000 rpm (20000 g) was then carried out. The clear supernatant liquid was precipitated twice with 0.1 N acetic acid at pH 5.8-6.1. Whereupon the centrifuged sediments were washed 3 times each with twice-distilled water and dissolved in 0.0033 M NaOH. The redissolved proteins were then dialyzed in order to eliminate the charging effect and make possible a determination of the diffusion constant at + 2°C. In experiment Pd 40c₂d dialysis was carried out for 24 hours with 0.01 M Na₂CO₃ and 0.08 M NaHCO₃. In experiments Pd 41-44 dialysis was conducted with 0.001 M Na₂CO₃ and 0.008 M NaHCO₃ (Figures 24-25). Steady rotation was applied during the dialysis. In the dd experiments, dialysis was carried out twice for 24 hours. For experiment 49/52 U₂ the polyheders were dissolved (5 mg/cm³) in 0.006 M Na₂CO₃ and 0.05 M NaCl. Then centrifuging was carried out for 30 min. at 16000 rpm (20000 g) followed by 2 precipitations with 0.1 N acetic acid and washed three times in water. After this the protein was dissolved in 0.005 M soda and 0.05 M NaCl and dialyzed under rotation and exclusion of air at + 2°C. One can see again from Figure 26 the exceptional sensitivity of the polyheder proteins, because in this case, under conditions which are otherwise completely identical with those shown in Figure 25, the doubled concentration of protein resulted in association and formation of the 18-19-Svedberg component.



The Lm larvae polyheders (5 mg/cm^3) were dissolved (to give a typical instance) in 0.005 M NaOH . The solution was then centrifuged for 15 min. at 16000 rpm (20000 g) and the protein precipitated 6 times with acetic acid (pH 9.5). After every precipitation the protein was washed 3 times with water. It was redissolved in $0.001 \text{ M Na}_2\text{CO}_3$ and 0.008 M NaHCO_3 , whereupon dialysis under rotation and exclusion of air was carried out at $+2^\circ$ (Figure 27).

The Bm polyheders (5 mg/cm^3) were dissolved in $0.005 \text{ M Na}_2\text{CO}_3$ and 0.05 M NaCl . After centrifuging had been carried out twice for 30 min. at 16000 rpm (20000 g), and the clear supernatant liquid had been precipitated with 0.1 N acetic acid, the sediment was washed 3 to 5 times with twice distilled water and redissolved in $0.0025 \text{ M Na}_2\text{CO}_3$ and 0.05 M NaCl . Dialysis under rotation and exclusion of air was then carried out for 16 hours at $+2^\circ\text{C}$. Figure 31 shows the diffusion curves of this polyheder protein, the crosses indicating the course of the theoretical curve. The good agreement between the curves indicates that the protein was uniform.

Table 1 lists the results obtained in 10 sedimentation measurements and 13 diffusion measurements carried out on the Pd polyheder protein. The Lm and Bm polyheder proteins were investigated for approximately the same number of times in the same manner. The average values of the determinations for the three polyheder proteins are given in Table 2.

To calculate the molecular weight, a precise determination of the partial specific volume V_0 of the non-solvated protein molecule was required in addition to this. This determination was carried out by the pycnometer method (see page 30) and resulted in a value of $V_0 = 0.736$ (i.e., a lower value than that reported earlier). After s_{20} , d_{20} , and V_0 had been determined, the molecular constants and the molecular weights of the three polyheder proteins could be calculated. They are listed in Table 2. The calculation was carried out according to equations which originated with Stocke and Svedberg. These equations were compiled recently by Markham, Smith and Lea [5] and are not discussed here further.

The very good agreement between the molecular diameters $2r_s$ and $2r_D$, which had been calculated from the independently measured s_{20} and D_{20} , as well as the agreement between the molecular weights M_s , M_D , and $M_{s,D}$ gives assurance to the effect that the M_0 values are correct. One must assume that the molecules deviate only very little from the spherical shape, because s_{20} is independent of changes in the concentration and f/f_0 is not much greater than unity when the fraction corresponding to the increase in friction due to solvation has been deducted. (See pages 31-36). All attempts made hitherto to crystallize the 12-Svedberg component with renewed formation of polyheders were unsuccessful. The polyheder protein precipitated in an amorphous state with or without dialysis of the alkaline solution, even when the latter was highly concentrated. Lowering of the pH, addition of different salts, application of room temperature or lowering of the temperature to 0°C did not help.

b) The 3- and 1.5-Svedberg Components Produced by Splitting.

The first component which results from the splitting of the polyheder protein is the 5-6-Svedberg component (Figures 17 and 18), to which one half of the molecular weight (approximately 140-180000) of the 12-Svedberg component must be assigned. It is very difficult to isolate this component in a uniform state as compared with the following, more stable splitting component, i.e., the 3-Svedberg component.

To isolate it, the Pd polyheders (5 mg/cm^3) were dissolved in $0.006 \text{ M Na}_2\text{CO}_3$ and 0.05 M NaCl , followed by precipitation with acetic acid (pH 5.9) washing three times with water, renewed solution in $0.1 \text{ M Na}_2\text{CO}_3$ and 0.05 M NaCl , and dialysis for 24 hours under rotation (Figure 28).

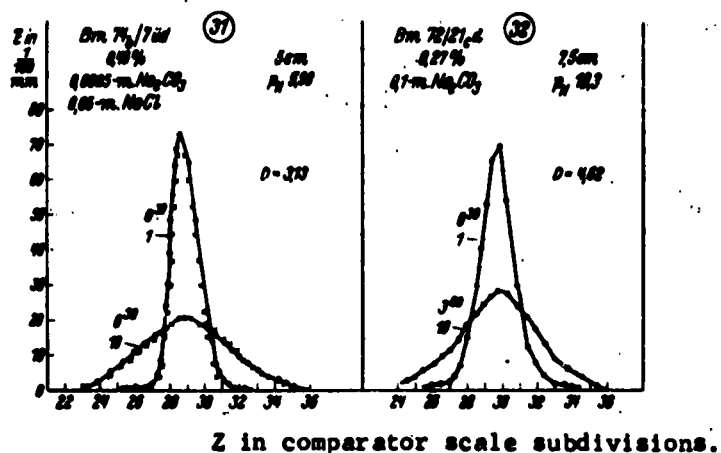
The 3-Svedberg component of the Lm polyheder protein could not be prepared because only a small quantity of the polyheder material was available.

TABLE 1. SEDIMENTATION AND DIFFUSION CONSTANTS OF THE
Pd POLYHEDER PROTEIN AT DIFFERENT CONCENTRATIONS

Experimental Solution Pd 49/	c%	Buffer	pH	s_{20}	D_{20}
40 c ₂ d	0.042	0.01-m. Na ₂ CO ₃ 0.08-m. NaHCO ₃	9.0	--	4.24
40 c ₂ d	0.063	0.01-m. Na ₂ CO ₃ 0.08-m. NaHCO ₃	9.0	12.19	4.04
40 c ₂ d	0.125	0.01-m. Na ₂ CO ₃ 0.08-m. NaHCO ₃	9.0	13.00	4.06
41 c ₂ d	0.041	0.001-m. Na ₂ CO ₃ 0.008-m. NaHCO ₃	8.9 9.3	12.44	4.28
41 c ₂ d	0.062	0.001-m. Na ₂ CO ₃ 0.008-m. NaHCO ₃	9.3	12.46	4.21
41 c ₂ d	0.124	0.001-m. Na ₂ CO ₃ 0.008-m. NaHCO ₃	9.2	12.66	4.16
43 c ₂ dd	0.042	0.001-m. Na ₂ CO ₃ 0.008-m. NaHCO ₃	8.75	12.31	4.20
43 c ₂ dd	0.091	0.001-m. Na ₂ CO ₃ 0.008-m. NaHCO ₃	8.75	12.60	4.16
44 c ₂ d	0.035	0.001-m. Na ₂ CO ₃ 0.008-m. NaHCO ₃	9.2	--	4.46
44 c ₂ d	0.059	0.001-m. Na ₂ CO ₃ 0.008-m. NaHCO ₃	9.1	12.72	4.31
44 c ₂ dd	0.055	0.001-m. Na ₂ CO ₃ 0.008-m. NaHCO ₃	8.75	--	4.11
44 c ₂ dd	0.082	0.001-m. Na ₂ CO ₃ 0.008-m. NaHCO ₃	8.8	12.59	4.0
49 52U ₂ d	0.410	0.005-m. Na ₂ CO ₃ 0.05-m. NaCl	10.35	12.75	4.13

Median values of s_{20} = $12.57 \pm 0.405 = \pm 3.1\%$

Median values of D_{20} = $4.18 \pm 0.23 = \pm 5.5\%$



The Bm polyheders (5 mg/cm^3) were (to give a typical example) dissolved in $0.005 \text{ M Na}_2\text{CO}_3$ and 0.05 M NaCl (or 7 mg/cm^3 in $0.015 \text{ M Na}_2\text{CO}_3$). This was followed by precipitation with acetic acid (pH 5.9), washing 3 times with water, and redissolving of the sediment in $0.1 \text{ M Na}_2\text{CO}_3$ (Figure 29). After this, dialysis was carried out for 24 hours with $0.1 \text{ M Na}_2\text{CO}_3$ to which NaCl (0.05 M) had either been added or not added (Figure 20 and 32).

The mean values of several s_{20} and D_{20} determinations of the first stable splitting component of the Pd, Bm, and Lm polyheder proteins are listed in Table 2. Under assumption of the same value for V_0 as for the 12-Svedberg component the values given in Table 2 for the molecular weights and the molecular diameters without and with solvation can be calculated. Because of the higher f/f_0 ratio one must assume that these products of splitting no longer have a spherical shape (cf. pages 137-139). The magnitude of the molecular weights indicates that the polyheder protein is first split into six parts, because the M_0 values (47250 and 60500) agree very well with the theoretical molecular weights for a one-sixth part (46000 and 63000).

The next, smaller splitting product of the polyheder protein can no longer be obtained by dissolution of the polyheders (7 mg/cm^3) in soda, but with a relative degree of uniformity only by solution in $0.015\text{--}0.5 \text{ M NaOH}$. This component is preserved intact after short boiling in 0.5 M NaOH (Figure 5,8). From the median values obtained in some sedimentation experiments one could calculate the values in Table 2 for the

TABLE 2. MOLECULAR CONSTANTS OF THE Pd, Lm, AND Bm POLYHEDER PROTEINS

		s_{20} in Sved- berg	D_{20} 10^{-5} cm ² /sec	V_0	V	M_0	M_D	M_s	M_{SD}	$2r_0$	$2r_s$	$2r_D$	f/f_0	ω
Principal Components	Pd	12.57	4.18	0.736	0.821	276 000	405 800	407 000	405 350	8.64	10.19	10.18	1.18	0.469
	Lm	12.78	3.50	(0.736)*	0.865	336 000	655 000	662 200	653 830	9.22	12.19	12.15	1.32	0.942
	Bm	12.85	3.12	(0.736)*	0.890	378 000	894 200	904 500	893 700	9.59	13.72	13.02	1.42	1.376
First Splitting Component	Pd	3.12	6.08	(0.736)*	0.899	47 250 (46 000)**	120 700	129 900	120 620	4.79	7.05	7.00	1.46	1.582
	Lm***	—	—	—	—	(56 000)**	—	—	—	—	—	—	—	—
	Bm	3.16	4.80	(0.736)*	0.933	60 500 (61 000)**	235 500	235 200	235 150	5.21	8.86	8.87	1.70	2.855
Second Splitting Component	Pd	1.43	8.58	(0.736)*	0.906	15 360 (15 300)**	42 530	44 700	42 522	3.30	4.97	4.96	1.50	1.771
	Lm	1.38	6.98	(0.736)*	0.938	18 270 (18 650)**	76 300	79 620	76 450	3.49	6.12	6.10	1.74	3.155
	Bm	1.49	6.80	(0.736)*	0.937	20 230 (21 000)**	82 700	84 020	82 460	3.62	6.18	6.265	1.73	3.092

* Not measured

** Theoretical molecular weights

*** The first splitting component of the Lm protein could not be prepared in a pure state

s_{20} = sedimentation constant according to equation: $s_{20} = \frac{1}{x\omega^2} \cdot \frac{dx}{dt}$

D_{20} = diffusion constant according to equation: $D_{20} = \frac{x^2_{wp}}{2t}$

V_0 = partial specific volume of the protein molecule in solution without hydration according to formula:

$$V_1 a = \frac{m_0 - (m-h)}{e_0 h} \quad \text{and} \quad (1 - V_0 e) = \frac{1 - w_1}{m} \frac{dm}{dw_1}$$

V = partial specific volume of the protein molecule in solution with hydration according to equation:

$$V = \left(e + \frac{162 \eta^3 N^2 \pi^2 D^2 s}{R^2 T^2} \right)$$

M_o = molecular weight without hydration according to equations:

$$M_o = \frac{R T s}{D(1 - V_o \rho)}$$

M_D = molecular weight with hydration according to equations:

$$M_D = \frac{R^3 T^3}{162 \pi^2 \eta^3 N^2} \cdot \frac{1}{V D^3}$$

M_s = molecular weight with hydration according to equation:

$$M_s = 9 \pi N (2V)^{1/2} \left(\frac{\eta s}{1 - V \rho} \right)^{3/2}$$

M_{sD} = molecular weight with hydration according to equation:

$$M_{sD} = \frac{R T s}{D} + \frac{R^3 T^3 \rho}{162 \pi^2 N^2 \eta^3 D^3}$$

$2r_o$ = diameter of the molecule without hydration according to equations:

$$2r_o = \left(\frac{6 R T V_o s}{\pi N (1 - V_o \rho) D} \right)^{1/2}$$

$2r_s$ = diameter of the molecule with hydration according to equation:

$$2r_s = \left(\frac{18 \eta V s}{1 - V \rho} \right)^{1/2}$$

$2r_D$ = diameter of the molecule with hydration according to equation:

$$2r_D = \frac{RT}{3 \pi \eta N D}$$

f/f_o = molecular friction resistance divided by the molecular friction resistance of spherical molecules of the same volume according to equation:

$$f/f_o = 10^{-8} \left(\frac{1 - V_o \rho}{D^2 s V_o} \right)^{1/2}$$

w = g of water combined with 1 g of protein according to equation:

$$w = \frac{V - V_o}{1/\rho - V}$$

molecular constants of the second stable splitting product of the three polyheder proteins. These values confirm the existence of smallest protein unit of approximately 17500 which was first detected by Svedberg. The values of the molecular weights indicate that the first splitting product of the polyheders split into three parts in the second cleavage (theoretical values 15300, 18600, and 21000; found 15360, 18270, and 21000). This means that the initial polyheder protein split into 18 parts to form the smallest protein moiety. According to X-ray small-angle diffraction measurements carried out by Prof. O. Kratky, the results of which will be reported in detail later, the dimensions of the smallest unit cell occurring in the Bm polyheders amount to $4.53 \times 2.8 \times 2.04 \text{ m}\mu$. From this follows a volume corresponding to a molecular weight of about 20200, which is in very good agreement with the calculated value given above. The high f/f_0 ratio indicates that the smallest splitting product already deviates to a considerable extent from the spherical shape, even when the high value of this ratio is corrected for the very sizeable degree of solvation ($w = 3 \text{ g H}_2\text{O}$).

As distinguished from the 3-Svedberg component, the 1.5-Svedberg component obtained by short boiling in 0.5 N NaOH aggregated on dialysis with water to glassy, transparent Pd polyheder-like formations with a size of up to 5μ . This has already been reported by Glaser [6]. It has not been established yet whether these unstable formations, which disintegrate readily, are genuine crystals.

3. Reconversion of the Splitting Product into the 12-Svedberg Component

After the Bm polyheders (7 mg/cm^3) had been dissolved in 0.1 M Na_2CO_3 and then precipitated with acetic acid and redissolved in 0.1 M Na_2CO_3 (Figure 29) and this had been followed by dialysis with 0.015 M NaHCO_3 and 0.005 M Na_2CO_3 (pH 9.4), the concentration of the 12-Svedberg component increased. This was accompanied by a slight increase in the sedimentation rate of the splitting product from 2.87 to 3.68 Svedberg, which was presumably due to formation of the 6-Svedberg component and overlapping of the values for that reason. On further dialysis with 0.015 M NaHCO_3 (pH 8.5), the concentration of the 12-Svedberg component increased considerably and the 3-Svedberg component was almost completely converted into the 5-Svedberg component (Figure 30). When further dialysis was conducted with 0.05 M NaHCO_3 (pH 8.7), the 3-Svedberg component was converted completely into the 5-Svedberg component and the sedimentation rate of the 12-Svedberg component increased from 12.7 to 13.2. This increase was presumably caused by a slight admixture of higher aggregation stages.

On the other hand, the 1.5-Svedberg component (Figures 5,8) could no longer be converted into the higher stages by dialysis with 0.015 M NaHCO_3 (pH 8.3); there was merely an increase of s_{20} to 1.66 Svedberg due to contamination with small quantities of higher aggregation products.

TABLE 3. RECORD OF DETERMINATIONS OF ACTIVITY

Number of Experiment	VBm	Protein Concentration	Number of Insects	Cause of Death		Imagos	Lethality from Polyheder Disease, %	50% Activity g of protein/insect
				Polyheder Disease	Other Causes			
782	73/35	2.4 • 10-8	20	20	--	--	100	1.6 • 10-10
783		10-9	20	20	--	--	100	
784		10-10	20	12	--	8	60	
785		10-11	20	2	--	18	10	
792	73/35 h	2.4 • 10-8	20	20	--	--	100	6 • 10-10
793		10-9	19	17	--	2	89.5	
794		10-10	19	4	--	15	21	
786		2.4 • 10-6	20	20	--	--	100	
787	73/35 g	10-7	19	17	--	2	89.5	3 • 10-8
788		10-3	20	4	--	11	45	
795		2.2 • 10-6	20	15	--	5	75	
796		10-7	20	10	--	10	50	
797	73/35 ü	10-8	20	1	--	19	5	2.8 • 10-7
798		7.5 • 10-10	20	19	--	1	95	
799		10-11	20	16	--	4	80	
300		10-12	20	10	--	10	50	
807	73/35 W	2.4 • 10-8	20	20	--	--	100	1.3 • 10-9
808		10-9	19	11	1	8	57.9	
809		10-10	18	3	2	15	16.6	

After a method for the reproducible separation and identification of the polyheder protein and of its splitting products had been developed, the activity of the virus could be investigated.

II. Experiments on Insects and Their Evaluation

To carry out an investigation of the activity, the test solution was prepared in 3-4 dilutions differing by powers of ten. Then 5 cm³ of every concentration were injected to 20 insects by means of a microinjection syringe [1]. Each of the 60 experimental insects was kept during the test period in "Zwoelfer" or Petri dishes in solitary confinement.

All insects which died were investigated by examining smears in a dark field (Leitz Ortholux microscope, immersion objective 1/12 a N. A. 1.32, dark field oil condenser N. A. 1.20). If no polyheders could be detected in an insect that had died, this insect was deducted from the total number of 20; to evaluate the test, only the results obtained on 19 insects were used. If the experimental insects developed to the imago phase, they were regarded as healthy without further investigation. The Bm and Pd larvae, which had been bred for three years at the laboratory, were free of polyheders in the third year of breeding. Injection to the Bm larvae took place approximately 12 days (middle of the fourth phase) before the expected time of pupation and injection to Pd larvae took place approximately 18 days before the expected time of pupation. For purposes of determination of the concentration, the quantity of protein in grams contained in the 5 cm³ experimental solution was taken as a basis. To determine the 50% point, the percentage of insects which died of polyheder disease was plotted along the axis of ordinates of a probability diagram against the virus concentrations plotted logarithmically along the axis of abscissae.

For every concentration, the allowable region corresponding to 68% of probability ($k = 1$) was drawn in according to Schelling [8]. Then the straight line which fitted best was drawn and the concentration for the 50% point was read on the diagram (cf. Table 3 and Figure 33).

III. The Virus Activity of the Polyheder Protein

Numerous infection tests carried out on several hundred Bm experimental insects showed that the activity of the polyheder protein, i.e., the 12-Svedberg component, is not very high and corresponds to a minimum dose of 6×10^{-7} to 4×10^{-8} g/insect [See Note], which is very surprising for a molecule as small as this.

([Note]: Dilution for these experiments was carried out with approximately 0.001 M Na₂CO₃-NaHCO₃ buffers or with water which had been brought with NaOH to the pH of the test solutions (8.5 - 10.0). The titers reported before [2], which were much higher, were in all probability due (aside from the small number of experimental insects which was used) to a latent virus infection present in the insects taken from a free habitat.)

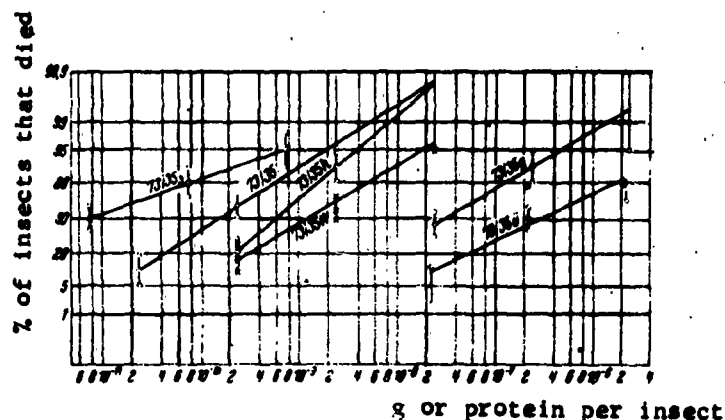


Figure 33. Statistical evaluation of experiments on insects

TABLE 4. ACTIVITY TESTS IN AN ULTRACENTRIFUGE FRACTIONATION EXPERIMENT (COMPARISON STANDARD HEMOCYANINE)

30 Min. 25000 rpm 4500 g	Hemocyanine		V Bm 72/24		Initial Activity g/insect	% of the Initial Activity
	c%	% of c	c%	% of c		
Initial Concentration (c)	1.48	100.0	0.60	100	$3.5 \cdot 10^{-8}$	100
Top	0.06	4.05	0.57	95	$2.8 \cdot 10^{-6}$	> 1.25
Middle	0.92	62.0	0.62	103	$2.8 \cdot 10^{-6}$	> 1.25
Bottom	6.98	470.0	0.72	120	$6.10 \cdot 10^{-8}$	58.3

After five reprecipitations followed by redissolving, the activity was reduced by about 10%. Frequently the activity was still lower and sometimes it even happened that solutions which had been found to contain a uniform 12-Svedberg component exhibited a lower activity than solutions which contained only splitting products.

In an attempt to explain this behavior, it was originally assumed that possibly the high salt and pH sensitivity of the polyheder proteins results in flocculation of the protein in the lymph to a certain extent (a phenomenon which can be easily observed), and that for this reason only a small, variable quantity of the molecules remains in a mono-disperse state. Under the circumstances, it was investigated whether or not addition of dispersion agents to the protein solution being injected increases the activity. Many experiments which involved the use of various substances such as mucin, tylose, periston, heparin, high-molecular sugar solutions, calcium chloride, peptone, etc. remained

unsuccessful. Then only two possibilities remained: either the 12-Svedberg component dissociated with a loss of activity at the high dilution involved, or the low virus activity was due to an admixture of an unknown component which had not been detected. Because the second possibility could be easily checked experimentally, a completely uniform sample of the 12-Svedberg component, which had been precipitated 5 times, was ultracentrifuged for 2 hours at 45000 rpm (150000 g). The layer which had formed and was approximately 5 cm high was divided into 4 layers with an equal volume. The individual layers were carefully siphoned off under sterile conditions by means of capillaries. Centrifuging reduced the concentration in the upper layer to about one half of that of the original solution and in the middle layer to about 4/5 of the original solution, while the concentration remained unchanged in the bottom layer. The fourth (lowest) layer could be brought into suspension only with great difficulty and in the form of large flakes; it was not investigated further. Testing of the activity of the upper three layers yielded rather surprising results, because within the concentration range investigated there was either no activity or an activity below 2×10^{-6} g/insect. On the other hand, the initial solution which had not been centrifuged showed an activity of 4.5×10^{-7} g/insect. This result invalidated to a considerable extent the assumption made originally that the active virus protein is identical with the 12-Svedberg component. Because of a shortage of food for the insects in the late fall of 1944, the experiments could not be repeated then.

The experiments were continued in the early summer of 1945. The 12-Svedberg component which had been reprecipitated only once (7 mg Bm polyheders/cm³ dissolved in 0.012 M Na₂CO₃, then precipitated and redissolved in 0.005 M Na₂CO₃) was centrifuged for 3 hours at 50000 rpm (180000 g). The result obtained was exactly the same as that obtained in the preceding year. Furthermore, the lowest (fourth) layer, which could be suspended only in a turbid state and in the form of large flakes, was also tested for its activity. Although the protein concentration in this layer was 3 times that of the initial solution, the activity was found to be even lower than that of the initial solution. The probability was that the infectious virus protein had a higher molecular weight.

Another fraction of separation by ultracentrifuging was carried out in which a solution of Bm polyheder protein which had not been precipitated (7 mg/cm³ in 0.012 M Na₂CO₃) was ultracentrifuged for only 30 min. at 25000 rpm (45000 g). Simultaneously, in the same experiment, hemocyanine from Helix pomatia (molecular weight of the principal component 8.7×10^6) was also centrifuged. Table 4 shows the concentration shifts for hemocyanine and the polyheder solution as well as the virus activity (See Note) of the initial solution which had not been centrifuged and of the 3 layers.

(/Note7: The solutions tested were diluted with 0.0025 M Na₂CO₃ and 0.0075 M NaHCO₃. It was established in earlier experiments /2/ that the activity was not reduced by ultracentrifuging. However, testing of the

activity was carried out with too small a number of insects and the tests were not reliable, because the insects were taken from a free habitat. Furthermore, insufficient attention was paid then to disturbances due to convection.)

One can clearly see that the virus activity had been centrifuged off completely, while the concentration of the 12-Svedberg component remained practically unchanged in the upper and middle layers. The virus protein molecule had to be larger than that of hemocyanine, because the virus protein was already absent in the middle layer, whereas the hemocyanine concentration in this layer was reduced to only 2/3. This surprising result made one suspect that already during the preliminary purification of the polyheder solution which, as has been mentioned before, was carried out by centrifuging for 15 min. at 16000 rpm (20000 g) in an Ecco-Blitz centrifuge, a part of the virus protein must have been separated out.

TABLE 5. ACTIVITY TESTS IN AN EXPERIMENT ON FRACTIONAL SEPARATION BY CENTRIFUGING (SUBSTANCE USED FOR COMPARISON TOBACCO MOSAIC VIRUS)

30 Min. 16000 rpm 20000 g	Tobacco mosaic virus		V Bm 73/3		Initial Activity g/insect	% of the Initial Activity
	c%	% of c	c%	% of c		
Initial Concentration (c)	0.50	100	0.54	100	$> 3.5 \cdot 10^{-8}$	> 100
Top	0.29	58	0.54	100	$2.7 \cdot 10^{-8}$	130
Middle	0.43	96	0.54	100	$2.9 \cdot 10^{-8}$	120
Bottom	1.25	250	0.56	104	$> 2.2 \cdot 10^{-8}$	> 159

The polyheder solutions for the following experiment (7 mg/cm^3 in $0.012 \text{ M Na}_2\text{CO}_3$) were therefore centrifuged for only 5 min. at 10000 rpm (7500 g) for purposes of purification and elimination of polyheder residues. In this case the supernatant polyheder solution was no longer clear, but showed a strongly opalescent Tyndall turbidity. In a subsequent test this polyheder solution was centrifuged for only 30 min. at 16000 rpm (20000 g) after addition of a tobacco mosaic virus solution. The concentration changes and the virus activity (See Note) of the 3 layers can be seen from Table 5: already the initial solution showed an activity which was so much higher that the 50% point could not be detected any longer under these experimental conditions.

(Note: The solutions used in the experiment were diluted with $0.0025 \text{ M Na}_2\text{CO}_3$ and 0.0075 M NaHCO_3 (pH 9.6-9.9).

The virus activity of the upper and middle layer was in the vicinity of 2.7×10^{-8} , whereas that of the lowest layer was either below 2.2×10^{-8} or could not be detected. Comparison with the concentration changes of the tobacco mosaic virus from layer to layer indicated that the molecule of the polyheder virus was presumably no heavier than that of the tobacco mosaic virus. An analytical test carried out by ultracentrifuging the same solution showed the presence of a weak and broad band after centrifuging at 10000 rpm (7500 g), which indicated that the virus protein must have a substantially higher molecular weight than that corresponding to the 12-Svedberg component.

At this stage an attempt was made to dissolve the polyheders in different alkali solutions at different values of pH and in the presence of different salts in such a manner that as high as possible a concentration of the high-molecular component would be achieved, because one was led to assume that under severe conditions of dissolution of the polyheders the high-molecular component could have been split. However, it was established after numerous experiments that the concentration and infectious capacity of the high-molecular component could not be raised (See Note), even when the polyheders were dissolved at 2° , and that the conditions under which the high-molecular component dissolved were the same as those determined for the 12-Svedberg component.

(/Note7: An attempt was finally made to dissolve Bm polyheders (5 mg/cm^3) in the stomach juice of Bm larvae, after this juice had been isolated from several hundred larvae by making them smell ether, as a result of which they threw up. The infectious capacity of these natural polyheder solutions (with a pH of approximately 9.1) was at the level of 9×10^{-10} g/insect lower than that of the most active artificial solutions. Ultracentrifuging resulted in only a very shallow layer of a high-molecular component, which had a low concentration.)

On the basis of a comparison between the areas under the sedimentation gradient curves, one could establish that the concentration of the high-molecular component amounted to only 5-10% of that of the 12-Svedberg component. From this point on, all polyheder solutions were centrifuged for only 5 min. at 6000 rpm (4000 g). Polyheder solutions with a lower alkalinity (5 mg/cm^3 in $0.0025 \text{ M Na}_2\text{CO}_3$ and 0.05 M NaCl) yielded broader or less uniform s-diagrams with an s_{20} of 2350-2550, whereas solutions with a stronger alkalinity (5 mg/cm^3 in $0.004 \text{ M Na}_2\text{CO}_3$ and 0.05 M NaCl) showed more uniform bands with an s_{20} of about 1540 Svedberg. An analytical layer separation test was carried out with one of those Bm polyheder solutions having a stronger alkalinity in order to find out whether the active virus protein was identical with the 1540-Svedberg component. First the solution was centrifuged at 10000 rpm for a sufficient length of time to make sure that the high-molecular band and the corresponding concentration gradient had reached the bottom of the cell (19.5 min. - cf. Figure 34). After the centrifuge had been stopped carefully, the contents of the cell were siphoned out very carefully until a layer of only 2 mm remained at the bottom (See Note).

(/Note/: No turbidity must rise from the bottom of the cell during this process. Even when no partition is used, mixing does not take place because of the very low diffusion constant.)

In a second experiment, centrifuging was carried out until about half of the cell was filled and the contents of the cell were again siphoned off until a layer of only approximately 2 mm remained.

TABLE 6. ACTIVITY TESTS ON A Pm POLYHEDER VIRUS SOLUTION AT DIFFERENT TIMES OF SEDIMENTATION

V Bm 73/35 Initial Poly- heder Solution	Sedimentation		Initial Activity g/insect	% of Initial Activity	s ₂₀ * Sved- berg	s ₂₀ Sved- berg
	Min.	Height to which cell was filled				
	0	0	$1.6 \cdot 10^{-10}$	100	--	1540
V Bm 73/35 .10000 rpm 7 500 g	19½ 18 + 1½	Full	$3.0 \cdot 10^{-8}$	0.54	1240	
	10½ 8 + 2½	Half Filled	$6.0 \cdot 10^{-10}$	27	1680	1600
V Bm 73/35 U	30 Min. 10000 rpm 7500 g and 30 Min. 2500 rpm 45000 g	Full	$2.8 \cdot 10^{-7}$	0.057	--	12.25

*Calculated from the decrease in activity (cf. Figure 38).

TABLE 7. TESTS OF ACTIVITY OF A Pd POLYHEDER VIRUS SOLUTION AT DIFFERENT TIMES OF SEDIMENTATION

V Pd 50/10 ₃ Purified Polyheder Virus	Sedimentation		c%	% of c	Initial Activity g/insect	% of Initial Activity	s ₂₀ * Sved- berg	s ₂₀ Sved- berg
	Min.	Height to which cell was filled						
	0	0	0.10	100	$1.5 \cdot 10^{-10}$	100	--	2450
10000 rpm 7500 g	10	Full	0.01	10	$5.0 \cdot 10^{-9}$	3	2200	3550 4290

*Calculated from the decrease in activity (cf. Figure 38).

Table 6 shows the concentration shifts and virus activity (See Note) (cf. Table 3 and Figure 33) of the initial solution, of two experimental solutions which had been centrifuged for different periods of time, 30 min at 25000 rpm (45000 g), and of the ultracentrifuged supernatant material

(12-Svedberg component). It also shows the sedimentation constants calculated in the customary manner from the rate of decrease of the concentration gradient and also from the decrease of the virus activity.

(Note7: The experimental solutions were diluted with 0.05 M NaHCO_3 (pH approximately 8.4)).

In connection with this, the activities corresponding to the sedimentation times (See Note) 19.5 min. and 10.5 min. (axis of abscissae) were indicated on the axis of ordinates (see Figure 38).

(Note7: This is the time from the first to the tenth or to the fifth exposure plus the time extrapolated back from the first exposure to the theoretical sedimentation starting point.)

Because the ordinates also represent the length of the cell (15 mm) or the sedimentation path, the sedimentation constant corresponds to the slope of the line times ω^2 . Figure 38 shows several solid straight lines labeled with the corresponding values of constants. These values were determined experimentally for the activities of 27%, 1680 Svedberg; 0.54%, 1240 Svedberg; and the median line corresponding to 1460 Svedberg. The agreement between the values leaves no doubt to the effect that the optically observed high-molecular component (median value 1570 Svedberg) is identical with active polyheder virus protein. Under the circumstances one had to abandon entirely the original assumption to the effect [1,2,3] that the main constituent of the polyheders, i.e., the 12-Svedberg component with a molecular weight of approximately 300000, was presumably identical with the infectious virus protein (See Note).

(Note7: Independently Glaser and Stanley [9] as well as Lauffer [10] also isolated a protein with a molecular weight of approximately 300000 and a molecular diameter of 10 μ . They also assumed, just as we did, that this protein was identical with the infectious virus protein. After completing the work described in this article, I received the original publications by Glaser and Stanley, according to which an infectious virus protein with a molecular weight of 300000 had been isolated from the blood of larvae infected with the polyheder disease. These publications will be discussed in greater detail elsewhere.)

Another line in Figure 38 shows that if a sedimentation constant of approximately 850 Svedberg is assumed for the infectious virus protein (first splitting product of the virus protein - see below), the activity must have decreased to 0% only after the expiration of 31.5 min. and must still approximately 40% at the expiration of 31.5 min [sic--this should be 21.5 min.].

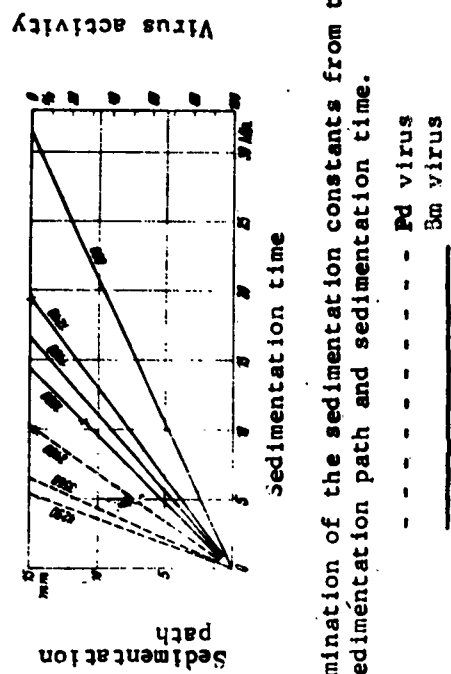
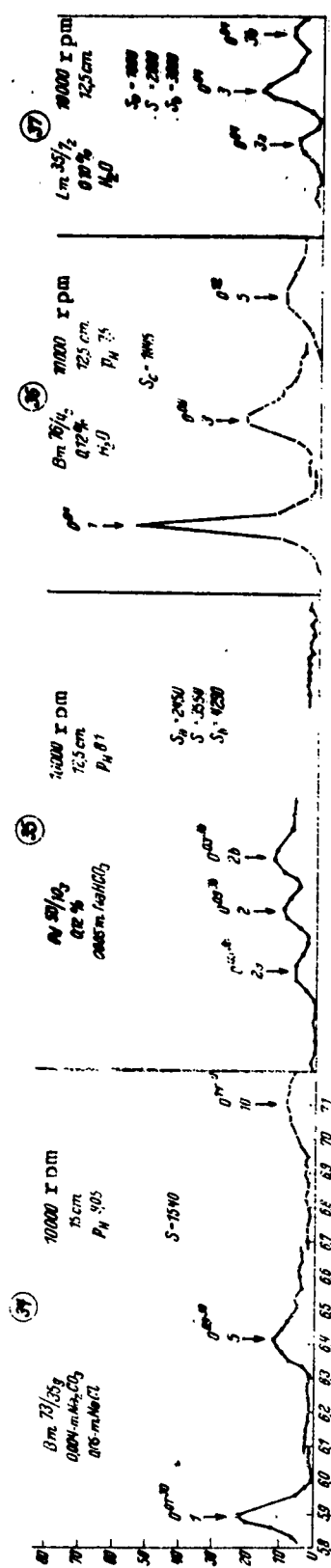


Figure 38. Determination of the sedimentation constants from the virus activity or the sedimentation path and sedimentation time.

A similar experiment with a purified (see below) Pd polyheder virus (Figure 35. and Table 7) and the data indicated by the dashed straight lines in Figure 38 demonstrated likewise that the observed three components of approximately 2450, 3550, and 4290 Svedberg (the concentration decreases parallel to the decrease in activity) may be identical with the infectious protein and that at least the 2450 Svedberg component is identical with it.

IV. The Polyheder Virus

1. Isolation and Properties

After it had been found in experiments on insects that the polyheders contained, in addition to the non-infectious polyheder protein (12-Svedberg component), the infectious virus in the form of a substance with a very high molecular weight, it was necessary to isolate the virus itself and to determine its characteristics. First separation of Bm polyheder virus from the polyheder protein (12-Svedberg component) was attempted by centrifuging. It was found advisable to centrifuge at 10000 rpm (7500 g) for about 1 hour, because in this case the sediments dissolve more readily than, for instance, after centrifuging at 25000 rpm (45000 g). This had already been established by Taylor in connection with the concentration of the influenza virus [11]. The clear polyheder protein solution could be poured off easily without admixture of the transparent, blue-white to light-brown gel-like sediment. Surprisingly the sediment (i.e., the Bm polyheder virus), as distinguished from the polyheder protein, could be dissolved most easily and uniformly in water and dissolved somewhat more slowly in very weak Na_2CO_3 -NaCl solutions or dilute solutions of NaHCO_3 , yielding sedimentation curves which became broader and less uniform. It was difficultly and non-uniformly soluble in 0.05 M NaCl and with great difficulty in an 0.01 M phosphate buffer, in which no monodisperse solution could be obtained. These characteristics were typical not only for the Bm virus protein, but also for the Pd and Lm viruses, although these two viruses were easily soluble in very dilute NaHCO_3 . Ca ions did not expedite dissolution, as had been found by Taylor [11] for the influenza virus; pyrophosphate was also ineffective. This great sensitivity of the polyheder virus to high salt concentrations, which we had already observed on the polyheder protein, easily produced blurring of the sedimentation curves or an increase of the sedimentation constants by reason of the formation of heavy aggregated particles. On the other hand, it was found that frequent washing with twice-distilled water made the virus protein less soluble and less uniform, so that very broad and flat sedimentation curves resulted.

TABLE 8. RANGE OF FLUCTUATIONS OF THE SEDIMENTATION
CONSTANTS OF Bm POLYHEDER VIRUS PROTEIN

a) Polyheder Protein + Virus Protein			
V Bm	Dissolved In	pH	s_{20} in Svedberg
73/3	0.002-m. Na_2CO_3 0.05-m. NaCl	9.1	1950
73/33	0.004-m. Na_2CO_3 0.05-m. NaCl	8.83	1825
73/34	0.004-m. Na_2CO_3 0.05-m. NaCl	9.05	1886
73/36	0.004-m. Na_2CO_3 0.05-m. NaCl	9.45	1800
73/37	0.004-m. Na_2CO_3 0.05-m. NaCl	8.74	1899
73/38	0.004-m. Na_2CO_3 0.05-m. NaCl	--	1879
73/39	0.004-m. Na_2CO_3 0.05-m. NaCl	9.57	1842
74a/4	0.004-m. Na_2CO_3 0.05-m. NaCl	9.3	1768
75/2	0.004-m. Na_2CO_3 0.05-m. NaCl	9.5	1800
75/4	0.004-m. Na_2CO_3 0.05-m. NaCl	--	1813
75/1	0.006-m. Na_2CO_3 0.05-m. NaCl	9.0	1865
75/8	0.006-m. Na_2CO_3 0.05-m. NaCl	9.7	1826
75/8g	0.006-m. Na_2CO_3 0.05-m. NaCl	--	1860
75/9	0.006-m. Na_2CO_3 0.05-m. NaCl	9.05	1753
75/10g	0.006-m. Na_2CO_3 0.05-m. NaCl	--	1961
75/11g	0.006-m. Na_2CO_3 0.05-m. NaCl	9.8	1902

continued on following page

Table 8 continued

Table 8 continued

V Bm	Dissolved In	pH	s_{20}^w In Svedberg	
76/1	0.006-m. Na_2CO_3 0.05-m. NaCl	10.05	1897	
76/3	0.006-m. Na_2CO_3 0.05-m. NaCl	9.7	1955	
75/5	0.008-m. Na_2CO_3 0.05-m. NaCl	--	1745	
75/6	0.016-m. Na_2CO_3 0.05-m. NaCl	--	1895	
Mean Value			1856	
b) Purified Polyheder Virus Protein				
V Bm	Dissolved In	pH	c%	s_{20}^w in Svedberg
73/35 ₂	0.05-m. NaCl	6.75	0.12	1779
73/36 ₂	H_2O	7.3	0.17	1750
73/37 ₂	H_2O	7.48	0.17	1949
73/37 ₂	H_2O	7.48	0.09	1885
73/38 ₂	H_2O	--	0.15	1895
73/38 ₂	H_2O	--	0.07	1963
73/39 ₂	H_2O	--	0.09	1892
75/4 ₂	H_2O	--	0.12	1880
74a 4 ₁	0.01-m. HCl 0.01-m. NaOH	6.18	0.12	1874
75/7 ₂	0.005-m. Na_2CO_3 0.05-m. NaCl	8.1	0.04	1955
75/7 ₂	H_2O	--	0.04	1906
75/7 ₂	0.005-m. Na_2CO_3 0.05-m. NaCl	8.1	0.04	1955
76/2 ₂	H_2O	--	0.12	1870
76/2 ₂	0.005-m. NaHCO_3	8.1	0.12	1926
76/2 ₂	0.005-m. NaHCO_3 0.05-m. NaCl	8.1	0.12	1956

continued on following page

Table 8 continued

V Bm	Dissolved In	pH	c%	s_{20} in Svedberg
76/2 ₃	H ₂ O	--	0.12	1905
76/3 ₂	H ₂ O	--	0.20	1890
76/3 ₂	H ₂ O	--	0.10	1932
76/3 ₂	H ₂ O	--	0.05	1959
76/4 ₂	H ₂ O	--	0.12	1845
76/4 ₂	H ₂ O	--	0.24	1754
76/5 ₂	H ₂ O	6.56	0.12	1855
76/8 ₂	H ₂ O	--	0.14	1809
Mean Value				1886
Mean Value = 1871 \pm 108 = \pm 5.8%				

For these reasons the following procedure for the isolation of the polyheder virus protein was found best. The polyheders (5 mg/cm³) were dissolved in the cases of Bm and Lm in 0.006 M Na₂CO₃ and 0.05 M NaCl and in the case of Pd in 0.008 M Na₂CO₃ and 0.05 M NaCl. Dissolution was carried out for 2-3 hours, whereupon centrifuging for 5 min. at 6000 rpm (4000 g) was conducted in order to purify the solution. Analytical ultracentrifuging of such Bm and Lm polyheder solutions (See Note) at 10000 rpm (7500 g) yielded already the virus protein component (Figure 34 and Table 8a), but this was not the case when a Pd polyheder solution was treated (20 cm along the scale).

(Note 7: However, the sedimentation rate of these proteins is occasionally reduced by reason of charging effects due to the strong alkalinity of the solution.)

The turbid polyheder solution was then centrifuged for 1 hour at 10000 rpm (7500 g) and the bluish-white to yellowish virus protein was suspended in twice-distilled water or some 0.005 M NaHCO₃ solution, bringing it to the volume of the clear polyheder protein solution which had been poured off. The virus protein dissolved rapidly (Tyndall turbidity). Then concentration was carried out for 1 hour at 10000 rpm (7500 g), the clear supernatant liquid was discarded, and the bluish-white virus protein forming the sediment was taken up in twice-distilled water or a very dilute NaHCO₃ solution, bringing the virus protein solution to 1/7 of the original volume. One hour later centrifuging was carried out for 5 min. at 4000 rpm (2500 g) to separate aggregated and undissolved components.

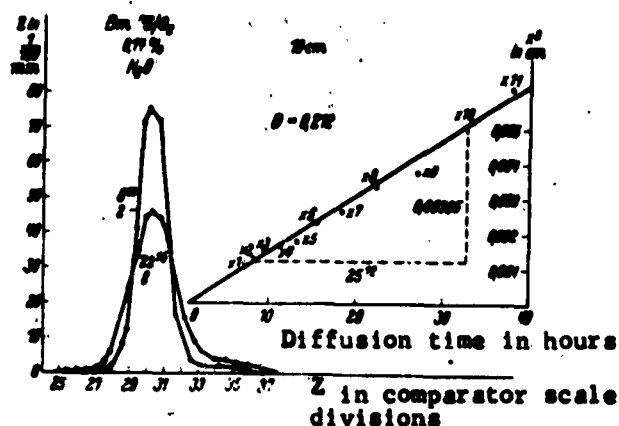


Figure 39

Analytical sedimentation tests carried out on such purified Bm virus protein solutions always yielded a single sedimentation limit with an s_{20} of 1871 ± 108 Svedberg ($\pm 5.8\%$) (Table 8, Figure 36.) One can see from Table 8 that s_{20} does not depend on the concentration. However, the relatively great width of the sedimentation curves (Figure 36) shows that the virus solution did not contain uniformly heavy or uniformly large particles. Of course, this uniformity could not have been expected at this particle size (see below).

The situation was different with Pd virus solutions purified in this manner. In this case, the numerous sedimentation tests which had been carried out did not yield a monodisperse sedimentation limit but showed in an entirely reproducible manner the presence of 3 components with s_{20} values of approximately 2500, 3100, and 4000 ± 200 Svedberg (Figure 35). It follows from this that Pd virus is larger and much less uniform than the Bm virus. The same applies to the Lm virus, the 3 components of which (Figure 37) showed s_{20} values of approximately 1800, 2810, and 3640 ± 100 Svedberg.

The diffusion constants of the Bm, Lm, and Pd viruses could be measured in the equipment described before [4] within 24-36 hours at concentrations of approximately 0.07-0.16% (Figure 39). The mean value of several measurements indicated that there is no concentration dependence for the Bm virus and that it has a D_{20} of $(2.15 \pm 0.065) \cdot 10^{-8}$ cm²/sec ($\pm 3\%$). The D_{20} found for the Lm and Pd viruses were only average values for the 3 components mentioned above. They amounted to approximately 1.75 (Pd) and 2.31 (Lm) $\times 10^{-8}$ cm²/sec.

The partial specific volume V_0 of the non-solvated virus particle was determined by the pycnometer at 0.770 ± 0.010 ($\pm 1.1\%$) for the Bm virus and at 0.740 ± 0.0032 ($\pm 0.43\%$) for the Pd virus (cf. Figure 40). V_0 showed no dependence on the concentration.

Because only small quantities of virus were available and because the investigation of the dependence on the concentration requires concentrations which are as small as possible, the following method of measurement was developed. A pycnometer was used which was based on the design proposed by Ostwald-Sprenger. At the upper end of a barrel-shaped glass vessel with a capacity of approximately 11 cm^3 were affixed 2 thick-walled capillaries with a lumen of approximately 0.3 mm , which were bent into the horizontal direction. The tips of both tube ends, which were approximately 3 cm long, had been polished. The air-free test solutions were carefully sucked into a weighing space which had a constant temperature of $19.9 \pm 0.05^\circ\text{C}$ and then weighed on a special Bunge microscale, after careful heating of the weighing space, so that within 1 hour a thermometer subdivided into 0.01 degree divisions showed a temperature of exactly 20.00°C in a control vessel which had a volume equal to that of the pycnometer. The minute droplets which came out of the tips of the capillaries evaporated and established automatically a well-defined, perfectly reproducible meniscus. By using this method, even very dilute solutions down to $4 \text{ mg}/11 \text{ cm}^3$ (0.04%) could be investigated if some degree of experimental aptitude was applied. As can be seen from Figure 40, there is a dispersion of the weight determinations by only about $\pm 50\%$, i.e., by $\pm 5 \times 10^{-4}\%$. More difficult is the exact determination of the concentration. If the exact N content of the salt-free, dried protein is known, it is simplest to calculate the concentration from the N content and the pycnometer volume, which can be determined exactly by weighing. If the test solution is free of salt, one can dry the complete contents of the pycnometer in a crystallization dish and then do the weighing.

All samples were dried in a high vacuum at room temperature, first over silica gel and then for several days over P_2O_5 . The calculation of V_0 was carried out according to equations given on page 13.

The virus protein solutions which had been purified by the method described above and concentrated by a factor of 7, had a concentration of approximately 0.15% , showed a strong bluish-white Tyndall turbidity, and had a virus activity of approximately 7.5×10^{-12} - 2.5×10^{-11} g/insect for Bm and approximately 10^{-10} g/insect for Pd. It was now investigated whether or not the activity of the virus proteins could be affected by a different preliminary treatment of the polyheders, of the polyheder solutions, and of the purified virus protein. It was found that the activity of the Bm polyheder solutions remained unchanged at approximately 1.5 - 3.0×10^{-10} g/insect, independently of the circumstance whether or not the polyheders were kept in distilled water or in dry air before dissolution and whether or not they were dried for a number of days in a high vacuum over P_2O_5 or frozen in water and kept in this condition for

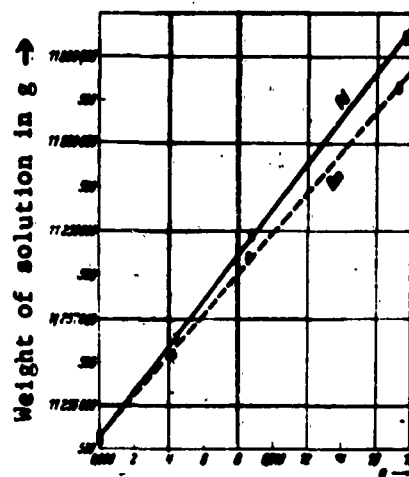


Figure 40. Relation between the weight of the virus solutions and the dry weight of the virus.

————— Pd virus
 ----- Bm virus

TABLE 9. PARTICLE CONSTANTS OF THE Bm, Lm and Pd POLYHEDER VIRUSES

		s_{20} in Svedberg	$D_{20} 10^{-7}$ ccm/sec	v_0	v	M_0	M_D
Polyheder Virus	Bm	1871	0.215	0.770	0.922	916,000,000	2,655,000,000
	Lm	2810	0.231	0.740*	0.871	1,136,000,000	2,260,000,000
	Pd	4021	0.175	0.740	0.891	2,146,000,000	5,140,000,000

*Not measured

		M_s	M_{sD}	$2r_0$	$2r_s$	$2r_D$	f fo	w
Polyheder Virus	Bm	2,720,000,000	2,651,000,000	130.7	197.9	198.1	1.51	1.90
	Lm	2,273,000,000	2,260,000,000	138.5	184.5	184.1	1.33	0.99
	Pd	5,395,000,000	5,098,000,000	171.5	243.2	243.2	1.42	1.36

hours. Freezing of a Bm polyheder solution and keeping it in a frozen condition for hours also did not affect the activity. On the other hand, the activity of a Bm polyheder solution in 0.05 M $NaHCO_3$ (a solution prepared for injections) dropped by approximately 12% after storage for 8 days at $+2^\circ$ and by approximately 20% in 31 days if the concentration of $NaHCO_3$ was 0.005 M .

When a solution of pure Bm virus was frozen, the virus precipitated quantitatively and only minor traces of N and P could be detected in the clear supernatant solution. The precipitated virus protein could not be redissolved under any conditions. The same applies to Bm virus obtained by drying its solution in high vacuum over silica gel. Attempts to redissolve the virus resulted in a turbid fine suspension (without the typical bluish Tyndall light dispersion), the virus activity of which had decreased by approximately 2% (after freezing) or 1% (after drying) (See Note).

(Note7: The results of these tests are somewhat uncertain, because the turbid suspensions could not be diluted without difficulty with 0.005 M NaHCO_3 .)

Similarly the activity of a pure Bm virus solution decreased by approximately 1% on being kept for 30 days at +2%. Addition of glycerine (1:1) reduced the activity by approximately 20% and by an additional 6% after the solution had been kept for 22 days at +2°. Dilution of Bm virus solution with ethyl alcohol or octyl alcohol in a ratio of 1:1 did not precipitate the virus. However, the activity of the virus was reduced by 2% in the case of ethyl alcohol and by approximately 33% in the case of octyl alcohol. On dilution of the virus solution with ether (1:1) the virus precipitated and lost its activity.

2. Particle Weight, Size, Shape, and Nature of the Polyheder Viruses.

a) On the Basis of Sedimentation and Diffusion Measurements

The particle weights and constants listed in Table 9 were calculated on the basis of determinations of the values of s_{20} , D_{20} and V_0 . The surprisingly good agreement between the particle diameters ($2r_s$ and $2r_D$ in Table 9) calculated on the basis of two independent measurements of s_{20} and D_{20} as well as the agreement between the particle weights M_D and M_s gives good assurance of the fact that the measured values were correct. The particle weight of the Bm polyheder virus amounts to 916×10^6 ; this virus molecule is therefore approximately 22 times heavier than the tobacco mosaic virus and approximately one-half as large as the elementary particle of the smallpox virus. Because f/f_0 amounts to 1.5, one must assume that the shape of the virus particles deviates from the spherical, even if a strong degree of solvation exists (see next chapter, Table 10). Calculation of the quantity of water which is bound to 1 g of the virus yields the value of 1.9 g. One must consider, however, that this value is too high because of the effect of deviation from the spherical shape (see next chapter and Table 10).

The particle weights of Lm and Pd viruses are of course only average weights of the three different rapidly-settling components mentioned above. The component with the highest concentration was taken as a basis for the calculation.

The Lm virus is somewhat larger than the Bm virus and presumably has, on the basis of its f/f_0 ratio, a more spherical shape; the degree of solvation is also smaller (See Note).

(Note): Because of the small quantity of Lm virus which was available, V_0 could not be measured, so that the Lm values (with the exception of s_{20} and D_{20}) are not quite certain.)

The Pd virus has the largest particle weight. However it is less strongly solvated and presumably has, because of the smaller f/f_0 value, a lower ratio between the axes than the Bm virus (cf. Table 10). However, this ratio is presumably higher than that of the Lm virus.

b) On the Basis of Electron Microscope Exposures

Because of the large particle weight and particle size of the polyhedral viruses, it was to be assumed that investigation by means of an electron microscope would yield further data. In Figures 41-44 (plate facing page 136) one can see very clearly elongated particles. These particles could be discerned without difficulty on approximately 20 different photographs of 10 different virus solutions. Comparison between the Pd and Bm virus particles shows that the latter (Figure 41) have a much more slender shape and are much more uniform with respect to the particle size and shape. This is in good agreement with the lower s_{20} and uniform sedimentation curve of the monodisperse Bm virus solution as well as the higher s_{20} of the much less uniform, "pauci-disperse" Pd virus solution (Figures 35 and 36). In the electron micrographs of the Pd polyhedral virus particles one sees in addition to the particles of different lengths and thicknesses some spherical formations, which are possibly elongated particles that do not stand perpendicularly to the field of vision. The measured lengths and diameters of Bm and Pd virus particles are correlated in Figures 45 and 46.

One can clearly see the much more extensive variations of the lengths and diameters in the case of Pd virus particles as compared with Bm virus particles (Table 10), which is in good agreement with the non-uniform, broadened-out sedimentation curves of Pd and the uniform curves of Bm virus. In comparison with other animal virus species and the tobacco mosaic virus [12], the Bm virus particles are very uniform, as distinguished from the Pd virus particles. If we calculate the weight of the virus particles from the mean values of the lengths and diameters (considering the particles to be rotation ellipsoids), we get the values listed in Table 10, which more or less agree with the particle weights calculated from s_{20} and D_{20} , considering the errors which may arise in the determination of diameters of dried-out protein particles from somewhat blurred electron micrographs. From the measured ratio of axes alone follow (cf. Table 10) the values 1.21 and 1.08 for f/f_0 , which are by approximately 0.32 lower than the f/f_0 values calculated from s_{20} and D_{20} . This difference must correspond to the solvation (cf. the preceding chapter).

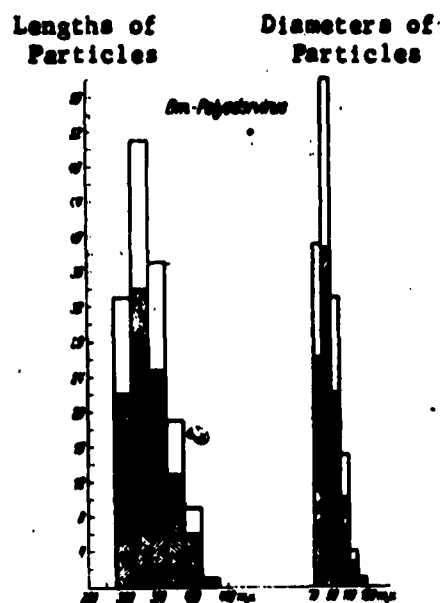


Figure 45. Length and diameter of 150 Bm virus particles.

The total height of the columns represents the number of particles, the cross-hatched part of the columns the percentage of these particles.

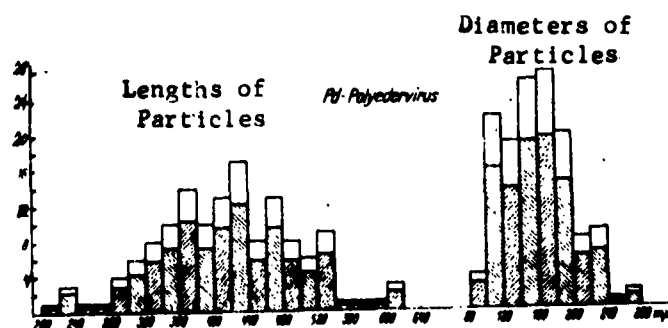


Figure 46. Length and diameter of 138 Pd polyhedral virus particles

The total height of the columns indicates the number of particles, the cross-hatched part of the columns the percentage of these particles.

TABLE 10. RANGE OF VARIATIONS OF THE POLYHEDER VIRUS PARTICLE DIMENSIONS

Poly- heder Virus	No. of Parti- cles	Mean Values in m/ μ		Deviations from the mean value			Particle Weight from Lengths and Diameters	Ratio of excess length to di- ameter	f from s a Length and Diameter
		Lengths $+3\sigma$	Diameters $+3\sigma$	Lengths Small- est	Lengths Large- est	Widths Small- est			
Bm	150	349.74 \pm 6.75	87.66 \pm 2.55	11	23	19	1098 \cdot 106	916 \cdot 106	4.4
Pd	138	415.2 \pm 20.82	160 \pm 10.0	52	49	50	4510 \cdot 106	2150 \cdot 106	2.6
									1.51
									1.21
									1.42
									1.08

Quite new and surprising is the bacteria-like shape observed, which is enhanced by the constriction in the middle of individual virus particles (Figures 42 and 43), so that one cannot classify the polyheder viruses among those of the rectangular type [13]. On the other hand, the apparent lack of a membrane and of an inner structure (See Note) precludes classification of these infectious agents as bacteria or rickettsia [14,15].

(/Note/; The electron-microscopic exposures were taken at a potential of 75 kv.)

However, the sensitivity to glycerin, alcohol, ether, and freezing must be regarded as an indication that the polyheder viruses are similar to organisms.

3. Experiments on the Splitting and Storage of the Polyheder Viruses

One must assume that even a very simple organism consists of different building blocks or molecules, which are not of the same weight and of which at least some occur in comparable concentrations. However, if a particle consists almost exclusively of protein or nucleoprotein which can be split completely into parts that have an equal weight, with the result that the total concentration of these parts corresponds exactly to the concentration of the intact particles (See Note), then we have a macromolecule which cannot be differentiated further and which does not exhibit the functions of an organism in the sense in which this is understood today.

(/Note/; This assumption cannot be verified by applying the inexact methods which are at our disposal at present.)

In the case of the polyheder virus an additional question arises as to whether the polyheder protein which has been characterized is a building block or a disintegration product of the infectious virus particle.

Under the circumstances preliminary experiments were carried out on the splitting of the polyheder virus. It was established that under the experimental conditions which were chosen splitting with an acid proceeded in a different manner than splitting with an alkali. The very weakly buffering Bm polyheder virus (0.12%) decomposed already in an 0.0025 M HCl solution (See Note), forming to some extent particles with an s_{20} of approximately 850 Svedberg, i.e., presumably fourth parts of the original molecule (molecular weight approximately 230×10^6).

(/Note/; Appropriate concentrations of buffer solutions, which do not yet produce any salting-out effects, are being tested at present.)

A pH of approximately 2.9 became established and the Tyndall turbidity became distinctively weaker. In an 0.005-0.01 M HCl solution, the total quantity of the polyheder virus underwent splitting (pH 2.7 or 2.5). In the solution, which became gradually clearer, a component with approximately 600 Svedberg was formed in addition to the 850 Svedberg component (Figure 47). The 600 Svedberg component must have corresponded to an eighth part of the original molecule (molecular weight approximately 115×10^6). The fourth and eighth parts of the virus were completely inactive in experiments on insects. In 0.05 M HCl solutions a pH of 1.8 was established, while the solution became more turbid. The 850 Svedberg component which had formed was much less uniform and its concentration was lower. At an 0.1 M concentration of the HCl, this component disappeared completely and the pH of the solution, which was much more turbid, became established at about 1.55.

In the splitting with caustic, the pH of an 0.025 M NaOH Bm polyheder virus solution (0.12%) jumps to approximately 11.3. The turbidity decreases, and the 1871 Svedberg component disappears, with the result that only badly defined, non-uniform splitting products can be detected. In 0.005 M NaOH there is a more clearly defined formation in a solution which becomes still clearer of a component with about 400-450 Svedberg, presumably twelfth parts of the original molecule (molecular weight 77×10^6). This component forms at a pH of 11.7. In 0.01 M NaOH, this component is represented very prominently (pH 11.9) and is already accompanied by a second component which is present in a somewhat lower concentration, has a sedimentation number of 220 Svedberg (Figure 48), and presumably constitutes a twenty-fourth part of the original molecule (molecular weight approximately 40×10^6). The solution of twelfth and twenty-fourth parts of the virus particles, which had again become clearer, had an activity amounting to only about 4% of that exhibited by the initial virus solution. This remaining activity was presumably due to the presence of some virus particles which had not yet been split. In an 0.05 M NaOH solution only the 220 Svedberg component remained (pH approximately 12.0). In an 0.1 M NaOH solution (pH about 12) this component disappeared and the almost clear solution contained components with approximately 3.4 and 5.2 Svedberg in the absence of any intermediate components (Figure 49). This corresponds to molecular weights of approximately 35000 and 70000.

Further work is being done on the splitting of the virus. It seems that splitting products with 12.5 Svedberg, which are characteristic for the polyheder protein, do not form. Their formation might be expected if the polyheder protein were a typical building block of the polyheder virus. If one dialyzes the virus splitting product suspended in an 0.01 M NaOH solution (which contains twelfth and twenty-fourth parts), rotating at $+2^\circ$ for approximately 60 hours and using 2.5 liters of twice-distilled water which is often changed, one finds that the

slightly turbid solution becomes highly viscous and that it yields in the ultracentrifuge a uniform component with a total concentration corresponding to that of the splitting products in the initial solution (Figure 50) and an s_{20} of about 1485 Svedberg.

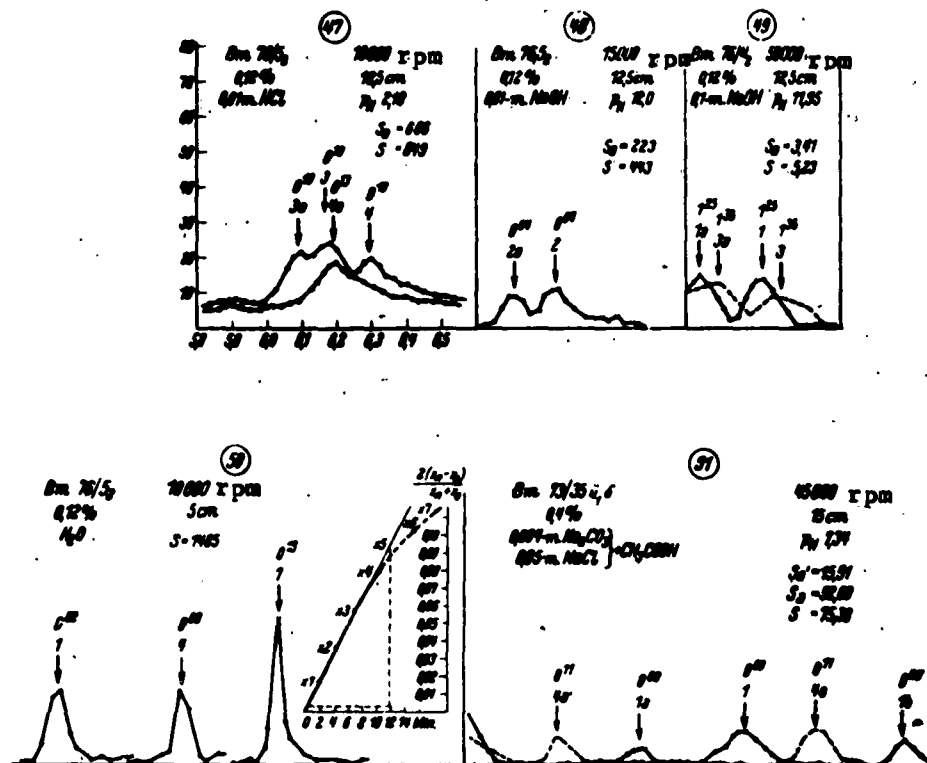


TABLE 11. THE NITROGEN AND PHOSPHORUS CONTENT OF THE POLYHEDERS AND THEIR CONSTITUENT PARTS

	Polyheder			Polyheder-Protein		
	Pd	Lm	Bm	Pd	Lm	Bm
% N x	14.72	15.26	14.73	15.44	15.62	15.16
% P xx	0.127*	0.175*	0.191	0.045	0.039	0.062
	-- 0.246		-- 0.243			
N/P	116-60	87	77-60	345	400	245

	1st Splitting Product 6/6		2nd Splitting Product 18/18		Polyheder Virus		
	Pd	Bm	Pd	Bm	Pd	Lm+	Bm
% N x	14.50	14.82	--	14.37	15.28	--	13.92
% P xx	0.05	0.07	--	0.07	1.33	--	1.34
N/P	290	212	--	205	11.50	--	10.40

x Micro-Kjeldahl determinations (precision + 1%); all samples were dried in high vacuum over P_2O_5 for several days.

xx Determined with a precision of + 2-3% by applying the strychnine molybdate method, which will be described elsewhere.

+ The Lm virus values could not be determined because only a small quantity of the substance was available.

* The values of the P-determinations on different polyheder preparations showed that there were variation in the P-content of the polyheders.

Because of the high viscosity, sedimentation was proportional to the time only in the beginning; it became slower at a later stage (Figure 50) and stopped almost entirely in the last quarter of the cell, with the boundary plane becoming increasingly well-defined. It was surprising that the sedimentation rate was almost the same as that determined for the infectious polyheder virus. This must have been just a coincidence. This artificially built up high-molecular substance, which also had the same P-content and N-content as the infectious virus protein, exhibited the same small activity which it had before dialysis, i.e., 4% of that of the initial virus solution. On being diluted with water in the ratio of 1:1, this substance decomposed into the initial virus splitting products. This also happened when the substance was tested for its activity.

If one dialyzes in the same manner the quarter and eighth parts of the virus which form in 0.01 M HCl solution (these splitting products have the same P and N content as the infectious virus, just as in the case of the alkali splitting products), flocculation of the solution which becomes turbid takes place and the solution remains totally inactive.

In connection with this, an attempt was made to aggregate the non-infectious polyheder protein to particles with a higher molecular weight. After 0.1 M acetic acid had been added gradually and in small portions to a clear and uniform, not reprecipitated Bm polyheder protein solution (principal component with a molecular weight of 370,000) until a pH of about 7.3 was reached and the solution just became turbid, several components formed from the polyheder protein main component which had s_{20} values of 52 and 75 Svedberg or values which were still higher (Figure 51). This corresponds to molecular weights of 2.5-3.5 million. These artificial high-molecular polyheder proteins proved to be completely inactive, however.

V. The Nature of the Polyheders and of Their Components

Before discussing the interrelationships of the polyheder components, we shall compare the conditions pertaining to the phosphorus and nitrogen content.

Desnuelle and co-workers [167] investigated in some detail the Bm polyheders and the Bm polyheder protein, which they regarded as the infectious virus protein on the basis of our earlier publications. They found that the Bm polyheders contain 14.5% of N, 0.248% of P, and 0.920% of S and that the Bm polyheder protein contains 15.0% of N, 0.127% of P, and 0.83% of S. On the basis of the P content they assigned to the Bm polyheder protein a minimum molecular weight of 97600, i.e., almost exactly a quarter of the value of 378000 found by us on the basis of the s and D determinations. Furthermore, they gave a very interesting compilation of the amino acids of the Bm polyheder protein on the basis of their experimental results.

Our own results are listed in Table 11. We found that the polyheders, the polyheder protein and the products of splitting of the polyheder protein as well as the polyheder virus have the same nitrogen content, whereas the phosphorus content varies greatly. The highest P content was found for the pure polyheder virus, which contained approximately 1.33% of P (See Note), and the lowest for the polyheder protein, which contained 0.039-0.062%, i.e., approximately half of the content found by Desnuelle, et. al.

(Note 7: On the basis of preliminary quantitative nucleic acid determinations with the Dische reagent, which had been carried out according to the method of F. B. Seiber and A. L. Dounce, the total quantity of phosphorus is presumably present in the form of thymonucleic acid phosphorus.)

It is interesting that the Bm virus has the same P content (with reference to the dry weight) as the Pd virus, although its N content is lower than that of the Pd virus, which possibly is the reason for the smaller specific volume V_0 (page 30) of the Bm virus. On the basis of the molecular weights M_0 found for the polyheder proteins (cf. Table 2), we find that a molecule of the Pd protein contains 3.9 (4), the Lm protein 4.2 (4), and the Bm protein 7.6 (8) atoms of P. Furthermore, it is noteworthy that in the splitting of the polyheder proteins (in 0.5 M NaOH) up to eight-tenths (mol wt. 15-20 000) of the total P remains. It is of course evident from this that in the splitting into sixths only in Bm can any splitting product contain a P atom; moreover, in the case of Lm, Pd and in the eight-tenth splitting products a P-atom is left over for only one part of these products.

A comparison of the high N/P ratio (Table 11-60) of the intact polyheders with the much lower values for the polyheder viruses (10.4 and 11.5) indicates that the polyheders cannot consist of virus protein only and that the polyheder protein does not in any case arise as a secondary splitting product of the polyheder virus following dissolution of the polyheders in alkali. We therefore have two different proteins with respect to their particle weight and their P or nucleic acid content. Both of these proteins are originally contained in the polyheders. A proof of this can be seen in Figure 52, which shows that one can discern by means of an electron microscope vacant sections having the approximate shape of a Pd virus particle in Pd polyheders which had partly dissolved. In other words, the virus particle has been dissolved out of these polyheders.

Because a more detailed characterization by physico-chemical methods was impossible and a relationship between the two proteins could not be established by other means, an attempt was made in collaboration with Dr. Friedrich-Freksa to establish a relationship by means of a serological investigation. A Bm virus protein solution (0.15%) which had been purified and concentrated as described above and a polyheder protein solution (0.15%), both of which had been derived from the same polyheder solution, were used for this purpose. The polyheder protein solution was purified for a second time by one hour of centrifuging at 25000 rpm (45000 g) in order to remove virus protein which may have been present. Dr. Friedrich-Freksa had already been able to establish before that even small quantities of the polyheder protein exert an extremely strong antigenic action. A rabbit was immunized with the polyheder virus and another rabbit with the polyheder protein by injecting them with 15. mg three times at intervals of 5 days. Seven days after the last injection, immune serum was taken from the animals and reacted with the homologous and non-homologous antigen. The immune serum of the polyheder virus formed a precipitate with the polyheder protein and the immune serum of the polyheder protein formed a precipitate with the virus. This clear result could be checked further by establishing that the immune serum of the polyheder virus, on being exhausted by precipitation with the polyheder

protein, yielded a second, distinct precipitation on addition of the polyheder virus. The whole test was repeated and gave the same result. The result of this experiment, which was carried out by Dr. Friedrich-Freksa with approximately 7.5 mg of the polyheder virus, demonstrates clearly that the polyheder protein is related to the polyheder virus serologically and the latter contains additional antigenically active centers.

Gratia and Paillot [17] were able to establish in 1939 that polyheder solutions, about the complex nature of which did not yet know anything, have no serological similarity with the lymph liquid and tissue extracts of silk worms. One must conclude on the basis of this that the polyheder protein is totally or at least partially a component part of the polyheder virus (See Note).

(Note7: It is also conceivable that the nuclei of the body cells of larvae, at which the polyheders are formed, contain very sensitive proteins, which actually are similar to the polyheder protein, but become denatured during extraction.)

Three possibilities then exist:

1. The polyheder protein is a component of the polyheder virus which can no longer associate to a large virus particle because it has been deprived of the nucleic acid.
2. The polyheder protein is a decomposition product of the virus protein, a view which is supported by its complete insolubility in water.
3. The polyheder protein is a product of a reaction which takes place in the host's cell [18] and crystallizes around the virus, forming the polyheders.

It is most likely that the polyheder protein resembles the so-called "soluble antigens" of other viruses, particularly the smallpox virus. In the case of the latter, one obtains similarly by careful splitting of the elementary particles with alkali a nucleoprotein and also a protein which is related serologically to the nucleoprotein.

To summarize, we established in our investigation that the water-insoluble polyheders, which according to work by Brill and von Kratky (unpublished) are genuine protein crystals, consist to the extent of about 82% of their weight (cf. Table 12) of a water-insoluble, P-poor non-infectious polyheder protein with a molecular weight of 276000 for Pd, 336000 for Lm, and 378000 for Bm and in addition to that contain approximately 5% of a water-soluble phosphorus and nucleic acid-rich, infectious polyheder virus with a particle weight of $1-2 \times 10^9$.

TABLE 12. DISTRIBUTION OF THE POLYHEDER COMPONENTS
WITH RESPECT TO WEIGHT AND THE N AND P CONTENT

	mg	Nitrogen		Phosphorus	
		mg	%	mg	%
Bm Polyheders	100	14.73	100	0.214	100
Undissolved Polyheder Residues	1.00	0.14	0.96	0.004	1.8
Polyheder Protein	82.20	12.42	84.40	0.023	10.7
Virus Protein	5.00	0.69	4.70	0.056	26.2
Undissolved Virus Protein	0.22	0.03	0.21	0.003	1.4
Low-Molecular Fractions, Losses During Precipitation	11.58	1.45	9.73	0.129	60.2

One can see from Table 12 that the polyheder proteins contains approximately 85% and the virus protein approximately 5% of the total nitrogen. On the other hand, the polyheder protein contains only 11% of the total phosphorus as compared with a content of about 26% in the virus protein. The missing 10% of N are lost in precipitation and washing, whereas the balance of 60% of P is recovered almost completely as dialyzable, free phosphate. The solution of the polyheders and the separation of the two proteins can be carried out only in weak alkali solutions and is accomplished best by centrifuging. According to the sedimentation and diffusion tests and the direct measurement of dimensions in electronmicrographs, the virus particles, which resemble bacteria as far as their shape is concerned, have a length of about 350 m μ and a diameter of about 87 m μ in the case of Bm larvae and the dimensions 415 and 160 m μ in the case of Pd larvae. Particles of this size are still discernible in the dark field of a visible light microscope, so that one may assume that the "clamidozoa" originally observed by Prowazek [18] in the illuminated field after staining are perhaps actually identical with the infectious virus particles, which have later also been described by Komarek and Breindl [19], Paillot and Gratia [20], and Letje [21]. The pictures published by me [17], which show minute vibrating particles inside the thin polyheder membrane or outside of the membrane after they have been released in the form of a cloud, presumably represent the polyheder virus itself or low aggregation stages of this virus rather than undissolved aggregation stages of the polyheder protein, as has been assumed originally. A rough estimate of the number of the vibrating particles, which become visible on solution in alkali, leads to a quantity of several hundred, which is in good agreement with respect to the order of magnitude with the values derived from the ratio of weights

and the number calculated on the basis of the particle weight. A further argument in favor of the assumption that these particles, which are barely visible in the dark field, are actually polyheder virus particles is the finding that when centrifuged solutions which have a low infectious capacity are observed under the microscope, only a few of these particles are seen as compared with the cloud-like swarms which are seen when non-centrifuged, active solutions are observed. This had already been established by Paillot and Gratia [207].

In the course of the investigation described, 434 analytical ultracentrifuge tests and 92 diffusion measurements were carried out during 4 years. Equipment which has been described elsewhere [47] was used for this purpose.

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Summary

By treatment with dilute alkali and centrifuging, the substance of the polyheders found in moth larvae infected with the polyheder disease could be separated into a water-insoluble protein and a water-soluble nucleoprotein. The two moieties could be differentiated chemically on the basis of their phosphorus content, which was low in the case of the protein and high in the case of the nucleoprotein. It is concluded on the basis of experiments on insects that the nucleoprotein rather than the protein, which had been mistaken for the virus in earlier work, must be regarded as the causative factor of the virus infection known as polyheder disease. The nucleoprotein is likened to the smallpox soluble antigen, which can be separated by similar methods from the smallpox virus.

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